AD_____

Award Number: W81XWH-06-1-0714

TITLE: Uncovering the Mechanism of ICI-Mediated Estrogen Receptor-Alpha Degradation

PRINCIPAL INVESTIGATOR: Mr. Angelo Casa

CONTRACTING ORGANIZATION: Baylor College of Medicine
                                      Houston, TX, 77030

REPORT DATE: October 2007

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
          Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
                                      Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE (DD-MM-YYYY) 1-OCT-2007	2. REPORT TYPE Annual Summary	3. DATES COVERED (From - To) 4 SEP 2006 - 3 SEP 2007
4. TITLE AND SUBTITLE Uncovering the Mechanism of ICI-Mediated Estrogen Receptor-Alpha Degradation		5a. CONTRACT NUMBER
		5b. GRANT NUMBER W81XWH-06-1-0714
		5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S) Mr. Angelo Casa E-Mail: casa@bcm.edu		5d. PROJECT NUMBER
		5e. TASK NUMBER
		5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Baylor College of Medicine Houston, TX, 77030		8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012		10. SPONSOR/MONITOR'S ACRONYM(S)
		11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
A cytoplasmic ER variant (lacking the NLS), expressed in MCF-7 cells, is resistant to ICI-mediated degradation up until 12hr. At 24hr, this protein may start to degrade, so temporal effects of ICI on cER degradation will have to be further explored. Interestingly, this cytoplasmic ER variant may actually be stabilized by ICI after 6hr of treatment. A rhodopsin-fused full-length ER-alpha (containing its NLS) that is sequestered at the plasma membrane might be degraded by increasing concentrations of ICI in MCF-7 cells. Furthermore, this RhER construct may be post-translationally modified by ICI but not by estradiol.

15. SUBJECT TERMS
ER-estrogen receptor alpha ; cER-cytoplasmic ER mutant ; rhER-membrane-tethered ER mutant; ICI-ICI182780 ; E2-estradiol ; NLS-nuclear localization signal

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT	b. ABSTRACT	c. THIS PAGE		11	19b. TELEPHONE NUMBER (include area code)

Table of Contents

INTRODUCTION

       Selective estrogen receptor down-regulators are thought to exert their effects by binding and degrading ERα; however, the mechanism of degradation is unknown. Our laboratory has shown that a cytoplasmic ERα mutant (cER) that lacks an NLS is completely resistant to ICI-mediated degradation in C4-12 cells. However, a rhodopsin-fused full-length ERα (containing its NLS) that is sequestered at the plasma membrane is degraded upon treatment with ICI in this cell line. **I hypothesize, therefore, that the NLS domain itself, and not merely the subcellular localization, is the critical determinant in ICI-mediated degradation of ERα**. I predict that the NLS serves as a region for interactions with other proteins that may assist in the degradation process.

BODY

1) Research Training Environment

The Breast Center at Baylor College of Medicine (BCM) provides a unique training environment with multiple opportunities for me to grow as a young research scientist. In the past year, I have taken full advantage of these opportunities and will outline my primary accomplishments here.

- completed and received an "A" letter grade in the Molecular Carcinogenesis course taught here at BCM
- beginning a course in Translational Breast Cancer Research, which is taught by faculty members of the Breast Center
- successfully completed my qualifying exam
- presented data in poster format at the graduate student symposium at BCM
- contributed a section to a text book chapter entitled "Insulin-Like Growth Factor Signaling in Normal Mammary Gland Development and Breast Cancer Progression" (in press)
- am primary author of a review article entitled "The Type-I Insulin-Like Growth Factor Receptor Pathway: A Key Player in Cancer Therapeutic Resistance" (in press); this article largely focuses on the role of the insulin-like growth factor pathway in mediating resistance to numerous cancer therapies, including endocrine therapies, such as ICI

2) Research Project

Preliminary experiments performed in C4-12 cells (an ER-negative variant of MCF-7 cells that my mentor has shown can be reconstituted with a fully functional ERα (1)) have shown that a cytoplasmic ERα mutant (cER) that lacks a nuclear localization sequence (NLS) is completely resistant to ICI-mediated degradation. However, a rhodopsin-fused full-length ERα (containing its NLS) that is sequestered at the plasma membrane is degraded upon treatment with ICI. **I hypothesize, therefore, that the NLS domain itself, and not merely the subcellular localization, is the critical determinant in ICI-mediated degradation of ERα**.

Specific Aim 1: Determine the role of nuclear localization, or the NLS, in ICI-mediated degradation of ERα

• *Confirm that cER is resistant to degradation by ICI in MCF-7 breast cancer cells*

One of my primary goals is to verify the degradation data in other breast cancer cell lines. Therefore, I used wild-type, ERα-positive MCF-7 cells stably expressing GFP-cER to determine if this cytoplasmic ERα mutant protein is also resistant to ICI-mediated degradation in a different cell line (Fig. 1). As expected, endogenous ERα is degraded upon treatment with either estradiol (E2) or ICI. However, while cER is strongly degraded by increasing concentrations of E2, the effect of increasing concentrations of ICI on cER protein expression appears to be minimal.

Thus, cER seems to be resistant to ICI-mediated degradation in MCF-7 cells as it is in C4-12 cells.
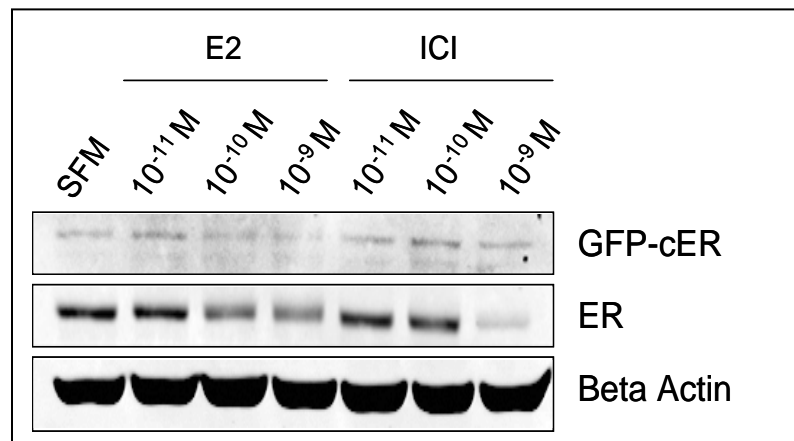
Figure 1. cER is partially degraded by ICI in MCF-7 cells. MCF-7 cells stably expressing cER were starved in serum-free medium (SFM) overnight and then either maintained in SFM or treated with increasing concentrations of E2 or ICI for 13hr. Protein levels were detected by Western blot using antibodies against either ERα or beta-actin.

The initial experiments performed in C4-12 cells analyzed ERα protein levels after 8hr of treatment with ligand. The data shown here are from MCF-7 cells treated with either E2 or ICI for 13hr. This led me to examine whether there is any time-dependence on ICI-mediated degradation of cER. To explore this possibility, I performed a time-course experiment using the MCF-7-cER cells (Fig. 2). As expected, endogenous ERα is degraded by both E2 and ICI at all time points tested. Cytoplasmic ERα mutant is degraded by E2 as early as 6hr, and this degradation persists for 24hr. This contrasts with the pattern of ICI-mediated degradation of cER in these cells. At both 6hr and 12hr, cER is completely resistant to degradation by ICI. However, at the 24hr time point, there is, potentially, a slight reduction in the levels of cER. Therefore, the length of treatment with ligand may influence ICI-mediated degradation of cER in MCF-7 cells. Interestingly, at the 6hr time point, ICI may actually stabilize cER.
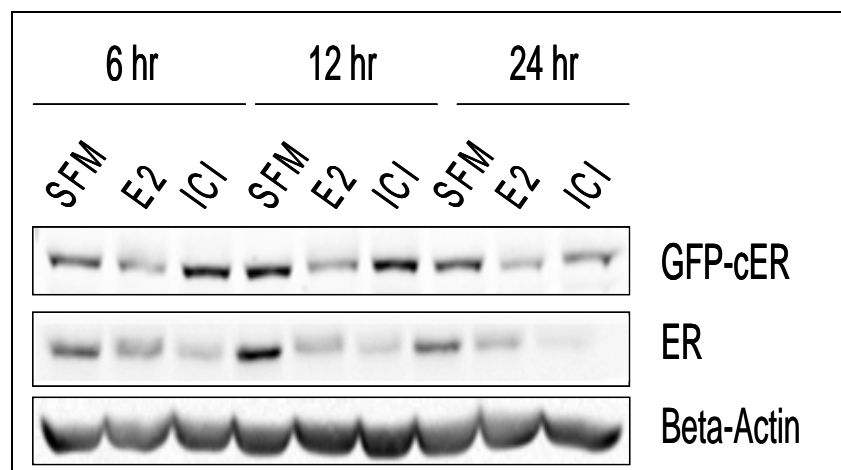

Figure 2. cER degradation by ICI is dependent on length of exposure to ligand. MCF-7-cER cells were starved in SFM overnight and then treated with either E2 (10^{-9}M) or ICI (10^{-9}M) for 6hr, 12hr, or 24hr. Protein levels were detected by Western blot, using antibodies against either ERα or beta-actin.

The data are clearly interesting, and I am in the process of repeating these experiments. I am focusing much of my efforts on understanding what occurs at the 24hr time point and later time points as well. It is critical to determine if the resistance of cER to ICI is merely temporal or whether it is a characteristic which persists regardless of the length of treatment.

• *Confirm that RhER, containing the NLS, is degraded upon treatment with ICI in MCF-7 breast cancer cells*

To determine if the susceptibility of RhER to ICI-mediated degradation that we observed in C4-12 cells occurs in other breast cancer cell lines, I treated stably transfected MCF-7/RhER cells with increasing concentrations of E2 or ICI (Fig. 3). As expected, endogenous ERα is degraded by increasing concentrations of both E2 and ICI. The data obtained for RhER seem to be less conclusive. It appears that increasing concentrations of E2 result in decreased levels of RhER; the protein detected after E2 (10^{-9}M) treatment seems to be considerably lower than the protein detected under control SFM conditions (unfortunately, this blot has a mark at E2 10^{-9}M, but this does not represent RhER protein). It appears that RhER levels decrease as ICI concentration increases from 10^{-11}M to 10^{-9}M. However, the total level of RhER protein detected at 10^{-9}M seems quite similar to the total level of RhER protein detected under control SFM conditions. Thus, it is difficult to conclude whether RhER, containing the NLS, is in fact degraded by ICI. More experiments clearly must be performed in order to resolve this issue. I am in the process of repeating this experiment, as well as performing time course experiments to determine if there are any temporal changes that exist.
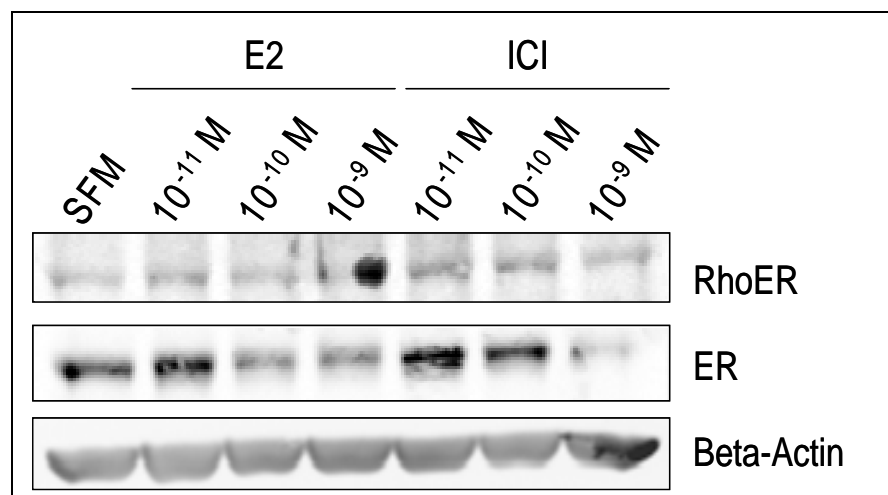
Figure 3. Degradation of RhER in MCF-7 cells. MCF-7-RhER cells were starved in SFM overnight and then treated with increasing concentrations of E2 or ICI for 8hr. Protein levels were detected by Western blot, using antibodies against either ERα or beta-actin

Interestingly, there appears to be a significant shift in molecular weight of RhER after treatment with 10^{-9}M ICI. Moreover, this shift does not seem to occur after treatment with 10^{-9}M E2. Thus, this lends support to the idea that E2 and ICI cause degradation by different mechanisms. The exact nature of this shift is currently unknown, but it may be due to poly-ubiquitination. I would like to examine this further in the future.

• *Characterize the role of the ERα NLS in degradation*

   To more completely characterize the role of the NLS domain of ERα in ICI-mediated degradation, I plan to force the cER mutant protein into the nucleus using the strong SV40 NLS. Furthermore, I plan to examine whether the ERα NLS can act as a degron by conferring degradation to a heterologous protein such as actin or GFP. I have only recently begun thinking about cloning strategies for these experiments. Some of the previously generated data must be carefully analyzed and more experiments need to be performed in order to determine the exact role of the ERα NLS in ICI-mediated degradation. I am continuing to plan my cloning strategy. I hope to resolve the current issues begin these further experiments within the next couple of months.

Specific Aim 2: ***Identify and characterize proteins binding specifically to ICI-bound ERα and, in particular, to the NLS domain***

   I have not yet begun to work on this aim, which, in the Statement of Work, was initially proposed for months 12-36.

KEY RESEARCH ACCOMPLISHMENTS

- Cytoplasmic ERα mutant protein, lacking the NLS domain, is resistant to ICI-mediated degradation following 12hr of treatment with the ligand in MCF-7 cells; in fact, cER may actually be stabilized by ICI at 6hr of treatment
- cER may start to be degraded by ICI after 24hr of treatment in MCF-7 cells; thus, the resistance to ICI-mediated degradation may be a temporal effect
- RhER might be degraded by increasing concentrations of ICI in MCF-7 cells
- RhER appears to undergo a shift in molecular weight after treatment with 10^{-9}M ICI; this shift does not seem to occur after treatment with 10^{-9}M E2

REPORTABLE OUTCOMES

- Casa, AJ, et al. "The Type-I Insulin-Like Growth Factor Receptor Pathway: A Key Player in Cancer Therapeutic Resistance" (in press)

CONCLUSION

   ERα is a critical marker for response to antiestrogen therapy. Although treatment with ICI is relatively successful, side-effects and the failure to respond in some patients is still a severe problem. In addition, indiscriminate degradation of ERα results in the loss of its beneficial qualities in bone and the cardiovascular system. A better understanding of the mechanism by which ICI mediates ERα degradation may lead to the development of new and better antiestrogen therapies to inhibit ERα action with less toxicity and greater specificity.

While the research that I have performed in the past year has provided some interesting results, it leaves many more questions that still need to be addressed. My immediate goal is to assess whether the effect of ICI-mediated degradation of our ERα mutant constructs is dependent on length of treatment with ligand. Once I can better understand this question, I will examine the response in other breast cancer cell lines, such as T-47D.

I have also attempted to expand my studies over the past year. My mentor's laboratory extensively studies the role of the insulin-like growth factor (IGF) signaling system in breast cancer. Significant cross-talk between the IGF system and ERα exists in breast cancer, and this interaction has been shown to be important for ERα stability. For example, a reduction in ERα expression is possible when aberrant and sustained growth factor signaling occurs (2). Specifically, 24hr of IGF treatment was shown to significantly decrease ERα protein expression in MCF-7 cells (3). To better understand this process, I have begun to use microarray and quantitative real-time RT-PCR to validate IGF- and E2-target genes. The data, to date, is promising and may provide us with target genes that are involved in resistance to endocrine therapies, such as ICI.

REFERENCES

1. Oesterreich, S., Zhang, P., Guler, R. L., Sun, X., Curran, E. M., Welshons, W. V., Osborne, C. K., and Lee, A. V. Re-expression of estrogen receptor alpha in estrogen receptor alpha-negative MCF-7 cells restores both estrogen and insulin-like growth factor-mediated signaling and growth. Cancer Res, *61:* 5771-5777, 2001.
2. Nicholson, R. I., Staka, C., Boyns, F., Hutcheson, I. R., and Gee, J. M. Growth factor-driven mechanisms associated with resistance to estrogen deprivation in breast cancer: new opportunities for therapy. Endocr Relat Cancer, *11:* 623-641, 2004.
3. Stoica, A., Saceda, M., Fakhro, A., Joyner, M., and Martin, M. B. Role of insulin-like growth factor-I in regulating estrogen receptor-alpha gene expression. J Cell Biochem, *76:* 605-614, 2000.

APPENDIX

BIOGRAPHICAL SKETCH
Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME **Angelo Casa**	POSITION TITLE **Graduate Student**
eRA COMMONS USER NAME	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing,*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Stony Brook University, Stony Brook, NY Baylor College of Medicine, Houston, TX	B.S. Ph.D. (in progress)	2001-2005 2005-present	Biochemistry Molecular Biology

Professional Experience

2003-2005   Undergraduate Research, Stony Brook University, Stony Brook, NY

Honors and Awards

2003   Golden Key International Honor Society, Stony Brook University, Stony Brook, NY

2005   Graduated Summa Cum Laude, Stony Brook University, Stony Brook, NY

2005   MCB Chair Scholar, Baylor College of Medicine, Houston, TX

2005   Rockwell Scholar, BRASS Organization, Baylor College of Medicine, Houston, TX

2007   Poster award winner at MCB Dept. Graduate Student Symposium, Baylor College of Medicine, Houston, TX

2007   Nominated to serve as a student representative on the Graduate Education Committee, Baylor College of Medicine, Houston, TX

Publications

1) Casa, AJ., et al. "Insulin-like Growth Factor Signaling in Normal Mammary Gland Development and Breast   Cancer Progression" *Breast Cancer: Prognosis, Treatment and Prevention (2nd Edition)* – in press

2) Casa, AJ., et al. "The type I insulin-like growth factor receptor pathway: a key player in cancer therapeutic resistance" *Frontiers in Bioscience* – in press